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## **Report Title**

### **ABSTRACT**

This project involves an Industry/University Cooperative Research Center consortium between The Ohio State University, the University of California, Davis, and North Carolina State University to assist in advancing food processing and packaging technology and will greatly benefit the US food industry and military personnel. This industry driven consortia center, CAPPS-Center for Advanced Processing and Packaging Studies, focuses on industrially relevant research directed toward coupling microbial life sciences with process and package engineering.

1    **Fourier-transform infrared microspectroscopy and multivariate analysis of *Bacillus***  
2    ***amyloliquefaciens* spore inactivation during pressure-assisted thermal processing**

3                    W. Ratphitagsanti, L.E. Rodriguez-Saona, and V.M. Balasubramaniam\*

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18    Department of Food Science and Technology, The Ohio State University, 2015 Fyffe

19    Road, Columbus, OH 43210-1007, USA

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21    \*Corresponding Author:

22    Tel: (614) 292-1732, Fax: (614) 292-0218, E-mail: [Balasubramaniam.1@osu.edu](mailto:Balasubramaniam.1@osu.edu)

## Abstract

Changes in spore composition of *Bacillus amyloliquefaciens* grown in different sporulation media (TSAYE and NAYE) as influenced by pressure-assisted thermal processing (PATP), high pressure processing (HPP), and thermal processing (TP) were investigated using Fourier-transform infrared spectroscopy (FT-IR). Additional experiments were carried out to compare and contrast the biochemical changes during single- and double-pulse PATP treatments with equivalent holding times. FT-IR spectra discriminated spore samples grown in two sporulation media as well as treated by different processing techniques (PATP, HPP, and TP). PATP and TP treatments caused major changes in calcium dipicolinate (CaDPA) structures as determined by FT-IR bands at 1381, 1415, and 1442  $\text{cm}^{-1}$ . These bands corresponds to the contribution of  $\text{COO}^-$  vibration of CaDPA chelate, the interaction of  $\text{Ca}^{2+}$  with  $\text{COO}^-$  group, and pyridine ring vibration of DPA (dipicolinic acid), respectively. Ratio of peak heights at 1381  $\text{cm}^{-1}$  and 1442  $\text{cm}^{-1}$  indicated the higher amount of CaDPA release by the double-pulse treatment. In addition, impact on amide bands (1540-1650  $\text{cm}^{-1}$ ) of protein were detected in TP and PATP treated samples. While FT-IR spectra were able to predict microbiological lethality for PATP (single-pulse) and TP treatments, it did not predict lethality changes during double-pulse PATP treatment. This may be possibly due to differences in mechanism of inactivation during single- and double-pulse treatment.

## Introduction

Consumers demand minimally processed shelf-stable low-acid foods, with better color, texture, appearance, nutritional values, with minimal or no preservatives. During traditional thermal processing, the product is heated by conduction or convection process and the severity of thermal treatment adversely degrade product quality and destroy heat sensitive ingredients. The food industry is investigating alternative sterilization technologies that can meet consumer demand for minimally processed safe low-acid food products. Among the alternative sterilization technologies investigated, pressure-assisted thermal processing (PATP) gained industrial interest in the recent years. It has a potential for manufacturing shelf-stable low-acid foods such as soups, mashed carrots, coffee, and tea ([Balasubramaniam et al., 2008](#)). In February 2009, its application for sterilization of low-acid shelf-stable products was approved by U.S. Food and Drug Administration (FDA) ([Anonymous, 2009](#)). The process preserves low-acid foods by using a combination of pressure (500-700 MPa) and temperature (90-121°C) over a short holding time (<10 min). One of the unique advantages of this technology is its ability to provide rapid and uniform temperature increase in the treated food as a result from heat of compression. Expansion cooling also occurs upon depressurization. This limits severity of thermal effect and provides superior product quality.

Application of pressure treatment at ambient temperature effectively inactivates variety of vegetative pathogenic and spoilage microorganisms. On the other hand, pressure in combination with heat is needed for bacterial spore inactivation. Number of earlier studies documented the effectiveness of PATP on inactivation of various bacterial spores including *Bacillus subtilis*, *B. amyloliquefaciens*, *Clostridium botulinum*, *C.*

69 *sporogenes*, and *Geobacillus stearothermophilus* (Margosch et al., 2006; Reddy et al.,  
70 2006; Ahn et al., 2007; Zhu et al., 2008; Akhtar et al., 2009; Bull et al., 2009). Pressure  
71 pulsing or cycling reported to further enhance spore lethality (Hayakawa et al., 1994;  
72 Meyer et al., 2000). Ratphitagsanti et al. (2009) reported that *B. amyloliquefaciens* TMW  
73 2.479 Fad 82 lethality was enhanced by 2-4 log CFU/ml when double-pulse treatment  
74 was employed over single-pulse treatment at 600 MPa-105°C for equivalent holding  
75 times. Very limited studies have been conducted to evaluate the mechanistic factors  
76 contributing to enhanced lethality during double-pulse treatment.

77 Advances in FT-IR spectroscopic instrumentation and multivariate analysis  
78 provide capability for rapid detection, identification, and characterization of spoilage and  
79 pathogenic microorganisms. FT-IR spectroscopy allows the simultaneous data collection  
80 from all frequencies, thus improving its sensitivity. Other advantages of FT-IR  
81 spectroscopy include simplicity, rapidity, and high throughput (Naumann 2000). Specific  
82 spectral patterns based on chemical and biological composition of samples are obtained  
83 and these unique biochemical fingerprints are the key for discrimination and  
84 identification among different biological specimen. The FT-IR microspectroscopy (FT-IR  
85 coupled with microscope) is effective to detect, identify, and classify various  
86 microorganisms at strain levels (Perkins et al., 2005; Schiza et al., 2005; Ngo Thi and  
87 Naumann, 2007; Brooke et al., 2008). Subramanian et al. (2006) employed attenuated  
88 total reflectance (ATR)-IR spectroscopy as an analytical tool to predict the viable spore  
89 counts after thermal and PATP treatments with correlation coefficient (r) of > 0.99 and  
90 standard errors of cross-validation ( $10^{0.2} - 10^{0.5}$  CFU/ml). The authors also found  
91 correlation between PATP spore resistance and calcium dipicolinate (CaDPA) content of

the spores when five different bacterial strains were investigated ([Subramanian et al., 2007](#)).

The objective of this study was to employ ATR-FTIR microspectroscopy to differentiate biochemical changes of bacterial spores during the inactivation by pressure-assisted thermal processing (especially single- and double-pulse treatments), high pressure processing, and thermal processing.

## **Materials and Methods**

### **Spore production**

Spores of *B. amyloliquefaciens* TMW 2.479 Fad 82 was provided by M. Gänzle from Department of Agricultural, Food and Nutritional Science, University of Alberta, Canada. Activation and isolation of the bacterial culture was done by the 3-phase-streak plate method on trypticase soy agar supplemented with 0.6% yeast extract (TSAYE; Becton, Dickinson and Company, Sparks, MD). The plate was then incubated in an aerobic condition at 32°C for 24 h. An isolated single colony was selected and transferred to a test tube containing trypticase soy broth supplemented with 0.6% yeast extract (TSBYE; Becton, Dickinson and Company). The spore crops were grown in two different sporulation media ([Table 1](#)). The first batch of the spore crop was prepared by spread-plating the 100 µl portions of *B. amyloliquefaciens* culture on TSAYE supplemented with 10 ppm MnSO<sub>4</sub>·H<sub>2</sub>O (Fisher Scientific, Pittsburgh, PA) ([Ahn et al., 2007](#); [Ratphitagsanti et al., 2009](#)). The second spore crop was grown on nutrient agar supplemented with 0.6% yeast extract (NAYE; Becton, Dickinson and Company) and 10 ppm MnSO<sub>4</sub>·H<sub>2</sub>O (adapted from [Mazas et al., 1995](#) and [Cazemier et al., 2001](#)). The inoculated plates on

TSAYE were aerobically incubated at 32°C for 10-14 days, whereas those of NAYE were incubated for 3-5 days to obtain 95% sporulated population. The sporulation was verified by using a phase-contrast microscopy. The surface of inoculated plates was flooded with 10 ml of cold sterile deionized water and the spores were scraped with disposable plastic spreaders. The spore suspension was washed five times by differential centrifugation that ranged from 2,000 to 8,000 x g for 20 min each at 4°C. Spore pellets were resuspended in sterile deionized water to obtain  $\sim 10^9$  spores/ml inocula. The suspension was sonicated for 10 min (SM275HT, Crest, ETL Testing Laboratory, Cortland, NY) following by heat treatment at 80°C for 10 min to destroy any remaining vegetative cells. Both spore suspensions were stored in a 4°C refrigerator.

#### **Sample preparation for PATP, HPP, or TP treatment**

For PATP and HPP experiments, spore suspensions (0.2 ml) were inoculated in deionized water (1.8 ml) and packaged in sterile polyethylene pouches (5cm x 2.5cm, 01-002-57, Fisher Scientific) at inoculum levels of  $\sim 1.6 \times 10^8$  CFU/ml for TSAYE crop and  $\sim 2.5 \times 10^8$  CFU/ml for NAYE crop. The pouches were then heat-sealed by an impulse heat sealer (American International Electric, Whittier, CA). After manually mixed the sample content, all pouches were submersed into an ice-water bath (4°C). Samples were treated within 2 h after preparation.

Sample preparation for TP experiments were done by aseptically transferred 0.2 ml spore suspension of *B. amyloliquefaciens* and 1.8 ml sterile deionized water into an aluminum-TDT-tube to obtain the final spore concentration of  $\sim 2.3 \times 10^8$  and  $\sim 3.4 \times 10^8$  CFU/ml for TSAYE and NAYE crops, respectively. All aluminum-TDT-tubes containing



the spore suspension were kept in an ice-water bath (4°C) up to 2 h before thermal treatments.

### **Combined pressure-thermal treatment**

Combined pressure-thermal experiments were conducted using a microbial kinetic tester (PT-1 test unit, Avure Technology Inc., Kent, WA). The spore samples were treated to various combined pressure-thermal treatment conditions by adapting published methods reported earlier ([Ahn et al., 2007](#); [Ratphitagsanti et al., 2009](#)). The come-up time of the PT-1 unit was  $0.67 \pm 0.08$  min. The sample pouch was placed inside a 10-ml polypropylene syringe (model 309604, Becton, Dickinson and Company) wrapped with insulating material (Sports Tape, CVS<sup>®</sup> Pharmacy Inc., Woonsocket, RI). The rest of the syringe was filled with ~8 ml of water to ensure uniform temperature within the syringe during treatment.

For PATP treatments, the syringe containing spore sample was heated to a pre-processing temperature of 58°C using water bath (Isotemp 928, Fisher Scientific) for 2 min. Preheated syringe was immediately loaded into the PT-1 unit. The pressure chamber was suspended in 105°C propylene glycol (Houghto-Safe-620-TY, Houghton International Inc., Troy, MI) bath. The propylene glycol was also used as the pressure-transmitting fluid within the PT-1 unit. PATP experiments were conducted at 600 MPa-105°C up to 8 min pressure holding times. Additional experiments were conducted to compare the microbial efficacy of single- and double-pulse treatments at an equivalent pressure holding time of 3 min. During double-pulse treatment, samples were taken for microbial and FT-IR analyses after various stages (C<sub>1</sub>, D<sub>1</sub>, B<sub>2</sub>, C<sub>2</sub>, and D<sub>2</sub>; [Figure 1B](#)). Spore sample pouches subjected to pressure-thermal treatment were immediately

immersed into an ice-water bath to cool the samples. Microbial analysis was conducted on the same day of experiments, while FT-IR samples were kept in a refrigerator up to 24 h before the analysis.

High pressure processing experiments (600 MPa-35°C up to 70 min holding time) were also performed by using a similar approach to that of PATP samples. The pre-processing temperature for HPP spore samples was  $4.9\pm0.8^{\circ}\text{C}$ . An untreated spore suspension of each spore crop was used as controls.

### **Thermal treatment**

Thermal inactivation of spores was conducted at  $105^{\circ}\text{C}$ -0.1 MPa using a 35L heating bath circulator (NESLAB EX-35 Digital One, Thermo Fisher Scientific Inc., Waltham, MA). Bath oil (Temperature range: -7 to  $176^{\circ}\text{C}$ , O220, Fisher Scientific) was used as heating fluid. The aluminum-TDT-tubes containing spore samples were pre-heated for 2 min at  $58^{\circ}\text{C}$  water bath before further subjected to  $105^{\circ}\text{C}$  oil bath. Sample temperature was monitored by inserting a K-type thermocouple attached to a data logger into a control aluminum cell containing sterile deionized water without spores. The thermal come-up time to reach  $105^{\circ}\text{C}$  was 2.33 min. The tubes were removed from the oil bath after the come-up time and different holding times (up to 240 min) and then immediately immersed into an ice-water bath. Standard plate count method was used to enumerate spores surviving the thermal treatment as well as untreated spore suspensions on the same day of processing. Samples for FT-IR analysis were kept in a refrigerator and the FT-IR analysis was carried out within 24 h after thermal treatments.

### **Enumeration of spore survivors**

182 Spore samples grown on different sporulation media (TSAYE and NAYE  
183 supplemented with 10 ppm  $\text{MnSO}_4$ ) and processed by various treatments were  
184 enumerated as follows. Sample contents (1 ml) were serially diluted in 0.1% peptone  
185 water and then spread-plated in duplicate on TSAYE. After incubation at 32°C up to 72 h,  
186 the viable count of spore survivors were enumerated.

#### 187 **Fourier-transform infrared microspectroscopy**

188 FT-IR analysis of treated spore samples was modified from [Subramanian et al.](#)  
189 [\(2006\)](#) and [Männig et al. \(2008\)](#). Aliquots (500  $\mu\text{l}$ ) were centrifuged at 13,000 rpm and  
190 4°C for 4.5 min. After removing the supernatant, the spore pellet was washed with 100  $\mu\text{l}$   
191 sterile deionized water and re-centrifuged at the same condition. The pellet was then re-  
192 suspended with 5  $\mu\text{l}$  sterile deionized water, applied onto hydrophobic Neo-grid filter  
193 membrane (NGFM; Neogen Corporation, Lansing, MI). The NGFM filters with  
194 deposited spores were then vacuum-dried to form a thin film. Infrared spectroscopic  
195 studies were carried out by using an Excalibur 3500GX FT-IR microscopy spectrometer  
196 (Varian, Palo Alto, CA) in Attenuated Total Reflectance (ATR) mode. The spectra were  
197 collected from the wavenumbers of 4000 to 700  $\text{cm}^{-1}$  (mid-infrared) region with a total of  
198 128 scans at a resolution of 8  $\text{cm}^{-1}$ . The FT-IR spectrometer was equipped with a  
199 PERMAGLOW mid-IR source, an extended-range KBr beam splitter, and a deuterated  
200 triglycine sulfate detector. Aliquot from each treated sample were applied onto three  
201 individual spots on the membrane and two measurements were taken at different  
202 locations on each spot. At least two to five independent replications of PATP, HPP, and  
203 TP treatments were carried out resulting in 12-30 spectra per sample per treatment time  
204 (six spectra per replication).

## **Multivariate analyses**

Pirouette<sup>®</sup> (version 3.11, Infometrix Inc., Woodville, WA) comprehensive chemometrics modeling software was employed to transform the spectra to their 2<sup>nd</sup> derivatives using a Savitzky-Golay polynomial filter (five-point window), mean-centered, and vector-length normalized. Classification analysis of samples processed at various conditions was further analyzed using principal component analysis algorithm (soft independent modeling by class analogy; SIMCA). Principal component analysis extracts information from the data set onto few dimensions, which are accounted for maximum possible variance (Mark, 2001). The spectral wavenumbers and their associated functional groups responsible for the classification of the spores could be identified using the discriminating power plot based on the measure of variable importance by minimizing the difference between samples within clusters and maximizing those from different clusters (Dunn and Wold, 1995). Correlation between specific spectral information (900 – 1800 cm<sup>-1</sup> region) and spore survivor curve (obtaining from the standard plate count) were determined using partial least squares regression (PLSR), which utilized large number of dependent variables to predict the viable spores surviving the treatments. A nonlinear iterative partial least-squares (NIPALS) algorithm was employed. PLSR with cross-validation (iterative recalculation of the model omitting a different sample point each time) was used to test for the model sensitivity.

## **Statistical analysis**

Statistical Analysis System software (SAS 9.1, SAS Institute Inc., Cary, NC) was used for data analysis. Independent variables were treatment (PATP single- and double-pulse, TP, and HPP), holding time, and sporulation media (TSAYE and NAYE), whereas

log reduction ( $\Delta \log \text{CFU/ml}$ ) in response to the treatments served as the dependent variable. The data was analyzed by the SAS program with the general linear model (GLM) procedure. The mean comparison were evaluated with the Tukey's test at a 5% significant level ( $P = 0.05$ ).

## RESULTS AND DISCUSSION

### Pressure and temperature history during PATP pulsing treatments and spore lethality

**Figure 1** provides sample pressure and temperature history of single- and double-pulse treatment at 600 MPa-105°C for 3 min holding time. Process temperature of the single-pulse treatment was well maintained at 105°C as the external glycol bath was heated to the same temperature. Even though both single- and double-pulse treatments had an equivalent time of 3 min under pressure, double-pulse treatment had higher total treatment time (~ 5.4 min) which included pause time between two pulses (1 min) as well as an additional pressurization time for 2<sup>nd</sup> pulse (0.7 min). This longer treatment time during double-pulse may also lead to higher process temperature values during the 2<sup>nd</sup> pulse. For example, during 1<sup>st</sup> pulse holding time (1.5 min), the process temperature ( $105.5 \pm 0.4^\circ\text{C}$ ) was maintained. Upon depressurization of the 1<sup>st</sup> pulse, the temperature dropped to 77°C. Subsequently during pause time between pulses (D<sub>1</sub>-B<sub>2</sub>), spore sample temperature increased to ~78°C due to heat transfer from the surrounding glycol bath which was kept at 105.5°C. This resulted in higher process temperature of  $112 \pm 0.9^\circ\text{C}$  during the 2<sup>nd</sup> pulse holding time (C<sub>2</sub>-D<sub>2</sub>). It is further worth to note that the temperature history obtained during double-pulse treatment likely further influenced by pressure

equipment design parameters (such chamber volume, pressurization rate, chamber insulation characteristics etc). The current study utilized a pressure chamber volume of ~20 ml and had relatively faster pressurization rate (~14 MPa/s). Accordingly, care must be taken in extrapolating the results of this study to larger pilot scale equipments.

At the equivalent 3 min holding time, enhanced spore lethality were observed in the double-pulse treatment (Table 2). Spores grown from TSAYE and NAYE media provided additional 2.6 log reduction from the double-pulse treatment than that from the single-pulse PATP treatment (600 MPa-105°C for 3 min). It is worth to note that the majority of the spore inactivation during double-pulse treatment (Table 2) took place during the 2<sup>nd</sup> pulse holding time where the spores were subjected to 600 MPa-112°C treatment for 1.5 min.

### **Combined pressure-thermal resistance influenced by sporulation media**

Among the two sporulation media, differences in spore resistant property were observed. Spore crops grown on NAYE produced higher PATP and TP resistant spores.  $D_{105^{\circ}\text{C}-0.1\text{ MPa}}$  values of the spore crop grown on TSAYE and NAYE were  $28.1 \pm 1.2$  min and  $36.8 \pm 1.5$  min, respectively. Similarly, D values of PATP treated spores grown on TSAYE and NAYE media were  $1.0 \pm 0.1$  min and  $1.4 \pm 0.2$  min at 600 MPa-105°C.

It has been well-documented that sporulation media impact spore heat resistance (Cazemier et al., 2001; Mah et al., 2008). Figure 2A shows that FT-IR could differentiate the two spore crops based on their resistant properties as influenced by different sporulation media. The corresponding peaks differentiated the properties among these two spore crops were found at 1388 and 1577  $\text{cm}^{-1}$  from the discriminating power plot

(data not shown). These bands are associated to the stretching bands of COO<sup>-</sup> group of Ca-DPA chelate and the C-N vibrations of the pyridine ring, respectively. The discriminating power is a measure of variable importance (i.e., IR frequency), which contributes to the development of the SIMCA pattern recognition and classification (Dunn and Wold, 1995). Results indicated that different levels of Ca-DPA chelate being inherited in the spore cores during sporulation. Moreover, FT-IR could also be used to ensure consistency and reliability of pressure-thermal resistance of each new spore crop. Differences in resistant property among TSA YE grown spores from various spore crop preparation were observed (Figure 2B). This may facilitate the evaluation of process resistance of the untreated spore crops before being treated.

#### **Classification of bacterial spores treated by thermal and pressure-assisted thermal processing**

Hydrophobic grid membrane filters (HGMFs) overlaid on a selective medium was previously used to isolate a single colony of *Salmonella* serovars (Männig et al., 2008). In this current study, a protocol was developed for the classification of bacterial spores treated by various processing methods by combining a hydrophobic grid membrane filters with infrared spectroscopy (Subramanian et al., 2006). Use of the hydrophobic grid membrane enabled direct spectroscopic observation of the biochemical changes in treated spores. The membrane confined spores within the barriers of the grid, while vacuum drying helps to limit the interference from water absorption bands. This sample preparation method was simple and improved signal intensity.

Figure 3A and 3B provides a sample SIMCA model illustrating consecutive changes in PATP and TP treated bacterial spores. FT-IR spectroscopy was clearly able to discriminate changes in untreated spore samples against that of PATP or TP treated samples. In TP treated samples, the biochemical changes gradually occurred over 240 min holding time (Figure 3B). Based on interclass distance ( $\leq 3$ ), the treated samples with similar changes in biochemical properties were grouped together resulting various distinct clusters (Figure 3A and 3B). Interclass distances are Euclidian distances between centers of clusters, which could be used as an indicator in SIMCA classification model (Kvalheim and Karstang, 1992). In general, large interclass distances (above 3) demonstrate well separation among the classes. PATP resulted in rapid lethality over a short time. Similarity in biochemical composition of the treated spores was observed after 2 min-PATP treatment as indicated by the same cluster containing the treated samples from 2-8 min holding times (Figure 3A). As expected, pressure alone (600 MPa-35°C, up to 70 min) did not clearly distinguish the untreated and treated spore samples into groups since *B. amyloliquefaciens* spores were not inactivated at this condition (data not shown).

### **Biochemical changes associated with PATP treated spores**

The spectral wavenumbers and the associated functional groups that were responsible for the classification of the spores in SIMCA class projections were identified using the discriminating power plot (Figure 4). The higher the value of the discriminating power, the greater is the influence of that wavenumber in classifying the samples that are in the model (Lavine, 2000). In comparison to untreated control, PATP caused predominant changes in the region of 1384, 1415, 1446  $\text{cm}^{-1}$  in both TSAYE and NAYE



grown spores. The identified bands represent changes in dipicolinic acid (pyridine-2,6-dicarboxylic acid; DPA) structure, especially the contribution of  $\text{COO}^-$  vibration of CaDPA chelate ( $1384\text{ cm}^{-1}$ ), the  $\text{COO}^-$  stretching vibration in the presence of  $\text{Ca}^{2+}$  ( $1415\text{ cm}^{-1}$ ), and the DPA pyridine ring vibration ( $1442\text{-}1446\text{ cm}^{-1}$ ) (Byler and Farrell, 1989; Cheung et al., 1999; Goodacre et al., 2000; Perkins et al., 2005). DPA is always chelated with divalent cations, especially calcium ions in a 1:1 ratio as CaDPA. This unique component is only present in bacterial spores and it represents about 5-10% of the dry weight of *Bacillus* spores (Setlow et al., 2006).

Highly resistant PATP-treated NAYE grown spores showed additional discriminating power peaks at  $1350\text{ cm}^{-1}$  (absorption due to lipids) and  $1577\text{ cm}^{-1}$  (C-N vibrations of the DPA pyridine ring or  $\text{COO}^-$  group of acidic amino acid residues of spore proteins) (Cheung et al., 1999; Wolfangel et al., 1999; Sahu et al., 2006) (Figure 4).

NAYE grown spores also caused changes in the protein region, specifically at  $1635\text{ cm}^{-1}$  (amide I of  $\beta$ -pleated sheet of secondary proteins),  $1543\text{ cm}^{-1}$  (amide II involved stretching vibration of C-N groups), and  $1273\text{ cm}^{-1}$  (amide III band of proteins or CaDPA band) (Helm and Naumann, 1995; Cheung et al., 1999; Schiza et al., 2005).

Table 3 presents discriminating power values at selected CaDPA bands ( $\sim 1377\text{-}1384$ ,  $\sim 1411\text{-}1415$ , and  $\sim 1438\text{-}1446\text{ cm}^{-1}$ ) and the corresponding log reduction of bacterial spores grown in different sporulation media during PATP, TP, and HPP. In general, discriminating power values increased with increase in holding times, within the same treatment condition and sporulation media. Although this observation appeared to hold good in most cases, the variation in the band intensity during TP holding times was

also observed. This might be due to the heterogeneity of spore populations as well as variability in pressure-thermal histories during PATP and TP replications.

#### **Contrast between single- and double-pulse PATP treatments**

**Figure 5** compares the discriminating power in classification of bacterial spores grown in TSAYE and NAYE media and subjected to single- and double-pulse PATP treatments. In general, regardless of the growth media, both single- and double-pulse treatments showed similar changes on the bands associated to CaDPA chelate, in particular at 1377-1381, 1411, and 1435-1442  $\text{cm}^{-1}$  (**Figure 5**). However, single-pulse treatment produced significantly higher discriminating power values than double-pulse treatment. According to [Cheung et al. \(1999\)](#), the ratio of peak heights at wavenumbers 1379 and 1443  $\text{cm}^{-1}$  could be used as an indicator of the CaDPA levels in bacterial spores. The higher the ratio, the more the CaDPA exists in the spores. Presence of high levels of CaDPA chelate, the relatively low content of core water, and the saturation of spore DNA with a group of small acid-soluble proteins (SASPs) play major roles in spore resistance properties ([Setlow and Setlow, 1995](#)). Specifically, the DNA- $\alpha/\beta$ -type SASP complex was the primary contributor to spore thermal stabilities, while divalent cations ( $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ ) and DPA provide synergistic effect on stability protecting the spore DNA against heat ([Setlow et al., 2006](#)). Both sporulation media demonstrated higher ratio of peak height associated to  $\text{COO}^-$  vibration of CaDPA ( $\sim 1377\text{-}1381\text{ cm}^{-1}$ ) and pyridine ring vibration ( $\sim 1435\text{-}1442\text{ cm}^{-1}$ ) in the single-pulse treatment. The peak ratios of single- and double-pulse treatments were 3.0 and 2.5 for TSAYE grown spores, as well as 1.5 and 1.2 for NAYE grown spores, respectively. The ratio suggested less CaDPA release from

the single-pulse treatment than that of the double-pulse treatment, indicating less lethality at the equivalent holding time.

### **Biochemical changes associated with TP treated spores**

At 105°C-0.1 MPa, it took more than 70 min to obtain any measurable spore inactivation (1.1-2.5 log reduction) in both spore crops. After 180 min holding time at 105°C, TSAYE grown spores were inactivated to undetectable level and NAYE grown spores had about 4.7 log reduction. FT-IR analysis of the TP spores indicated that discrimination was largely influenced by the similar bands observed from the PATP treatment, specifically at 1384, 1411, and 1442  $\text{cm}^{-1}$  (Figure 6). Biochemical changes among particular samples could be gradually monitored (data not shown). Three distinct groups of PATP treated samples were classified based on their interclass distance. Samples were grouped together when the interclass distance was lower than 3. Differences observed between the untreated control and the 1<sup>st</sup> group of treated samples (0, 2, 5 min) was mainly associated with the interaction of the  $\text{Ca}^{2+}$  with  $\text{COO}^-$  groups and the stretching of  $\text{COO}^-$  group of CaDPA chelate (1419 and 1381  $\text{cm}^{-1}$ ). When comparing changes between the 1<sup>st</sup> group (0, 2, 5 min) and the 2<sup>nd</sup> group (30, 70, 120 min), it was evident that bands associated with clustering of samples were related to the DPA pyridine ring vibration ( $\sim 1446 \text{ cm}^{-1}$ ), which became very prominent for discrimination of the 2<sup>nd</sup> group (30, 70, 120 min) from the 3<sup>rd</sup> group (180, 240 min) (data not shown).

Figure 7 demonstrates the influence of specific PATP and TP treatments that yield comparable 3 log reduction to *B. amyloliquefaciens* TMW 2.479 spores (NAYE grown).

It required at least 120 min at 105°C-0.1 MPa to achieve ~3 log reduction, while 5 min holding time at 600 MPa-105°C PATP treatment provided similar result. Both PATP and TP specifically acted upon CaDPA chelate (1276, 1373, 1411, and 1612  $\text{cm}^{-1}$ ) and its pyridine ring (1438 and 1573  $\text{cm}^{-1}$ ) (Figure 7). FT-IR results supported that there were similar changes happening in the structural level of DPA and CaDPA among the two treatments at the same lethality. However, the bands obtained from PATP were clearly much higher in discriminating power values (~45,000 arbitrary units) than that from TP treatment (~14,000 arbitrary units). This indicated that the biochemical changes of bacterial spores taking place during PATP was at the greater intensity than TP.

#### **Quantification of spore survivors from infrared spectra and validation of PLSR models**

Surviving spore populations of *B. amyloliquefaciens* TMW 2.479 after PATP and TP treatments could be estimated by cross-validated PLSR models using spectral region (900-1800  $\text{cm}^{-1}$ ) (Figure 8-9). The leave-one-out cross validation generally removes one sample from the training set, performed PLSR on the remaining samples. Then, it predicts the log spore survivors from the left-out sample and sums up the error until the total samples in the training set is analyzed. Good correlation on spore survivors obtained from the standard plate count and the mid-infrared spectral regions was found for both PATP and TP treatments (Figure 8-9). High coefficients of correlation ( $r > 0.96$ ) and low standard errors of cross-validation ( $\text{SECV} \sim 10^{0.16} - 10^{0.26}$  CFU/ml) were obtained from all PLSR models.

To verify whether or not single- and double-pulse treatments followed similar mechanism of inactivation, the developed PLSR model based on single-pulse PATP treatment was used to predict the spore survivors during various stages of the double-pulse treatment. Figure 5.10 shows the predicted spore survivors by FT-IR spectra and the experimental values obtained from the standard plate count during each stage of double-pulse treatment of *B. amyloliquefaciens* TMW 2.479 spores grown on different sporulation media. FT-IR spectra microbial lethality model based on single-pulse could not predict double-pulse lethality changes during 2<sup>nd</sup> pulse pressure holding time (D<sub>2</sub>), possibly due to differences in respective spore inactivation mechanisms. It is further possible that some of the biochemical changes were not detected during double-pulse treatment by FT-IR microspectroscopy. The single-pulse based PATP lethality model was successful in predicting (< 0.6 log CFU/mL) up to C<sub>2</sub> (2<sup>nd</sup> pulse come-up time; Figure 5.1.B and Figure 5.10) only. More studies are needed using advanced high resolution spectroscopic techniques such as Raman to further understand the nature of biochemical changes under these conditions.

## Conclusions

A study was conducted to investigate PATP, HPP, and TP treatment effects on biochemical changes of *B. amyloliquefaciens* spores. Both PATP and TP caused rise of CaDPA bands at approximately 1384, 1415, and 1442 cm<sup>-1</sup>. The intensity of these bands in general increased with increasing treatment times. For an equivalent log reduction, PATP showed higher intensities than that of TP. Changes in lipids and polypeptides were also evident in PATP-treated highly resistant NAYE grown spores. Release of CaDPA

from the spore core served as a key component indicating the inactivation of *B. amyloliquefaciens* TMW 2.479 spores. FT-IR spectra of the bacterial spores were not only influenced by the processing conditions, but also by the sporulation media.

#### **Acknowledgments**

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Table 1. Approximate formula per liter of media used for sporulation

Media component	Amount (g/L)
Difco <sup>TM</sup> Tryptic soy agar (TSAYE)	
Pancreatic digest of casein	15
Papaic digest of soybean	5
Sodium chloride	5
Agar	15
Yeast extract	6
Difco <sup>TM</sup> Nutrient agar (NAYE)	
Beef extract	3
Peptone	5
Agar	15
Yeast extract	6

Both media were supplemented with 10 ppm MnSO<sub>4</sub> .  
Source: Becton, Dickinson and Company (Sparks, MD)

Table 2. Log reduction of *Bacillus amyloliquefaciens* TMW 2.479 spores after single- and double-pulse treatments at 3 min equivalent pressure holding time

Total treatment time*	Log reduction (log CFU/ml)	
	TSAYE grown <sup>†</sup>	NAYE grown <sup>†</sup>
Single-pulse		
0.7 min (B-C; after come-up time)	0.2±0.3 <sup>A</sup>	0.3±0.1 <sup>A</sup>
3.7 min (B-D; after holding time)	-3.2±0.1 <sup>E</sup>	-2.5±0.3 <sup>D</sup>
Double-pulse		
0.7 min (B <sub>1</sub> -C <sub>1</sub> ; after 1 <sup>st</sup> pulse come-up time)	0.2±0.3 <sup>A</sup>	0.3±0.1 <sup>A</sup>
2.2 min (B <sub>1</sub> -D <sub>1</sub> ; after 1 <sup>st</sup> pulse)	-1.5±0.2 <sup>BC</sup>	-1.1±0.2 <sup>B</sup>
3.2 min (B <sub>1</sub> -B <sub>2</sub> ; after pause time)	-1.6±0.2 <sup>C</sup>	-1.2±0.1 <sup>BC</sup>
3.9 min (B <sub>1</sub> -C <sub>2</sub> ; after 2 <sup>nd</sup> pulse come-up time)	-2.2±0.3 <sup>D</sup>	-1.6±0.3 <sup>C</sup>
5.4 min (B <sub>1</sub> -D <sub>2</sub> ; after 2 <sup>nd</sup> pulse)	-5.8±0.3 <sup>G</sup>	-5.1±0.3 <sup>F</sup>

\*See Figure 1A and 1B for nomenclature. Total processing time includes pressure come-up time, pressure holding time, and depressurization time.

<sup>†</sup>Initial population of untreated controls: TSAYE -1.6 x 10<sup>8</sup> CFU/ml; NAYE - 2.5 x 10<sup>8</sup> CFU/ml.

Means with the same letter are not significantly different.

Table 3. Discriminating power values at bands associated to CaDPA structures and log reduction corresponding to treatments.

Treatment condition and holding time	TSAYE grown spores					NAYE grown spores				
	Wavenumber (cm <sup>-1</sup> )			Ratio of peak height $\frac{1384 \text{ cm}^{-1}}{1442 \text{ cm}^{-1}}$	$\Delta \log \text{ CFU/ml}$	Wavenumber (cm <sup>-1</sup> )			Ratio of peak height $\frac{1384 \text{ cm}^{-1}}{1442 \text{ cm}^{-1}}$	$\Delta \log \text{ CFU/ml}$
	~1377-1384	~1411-1415	~1438-1446			~1377-1384	~1411-1415	~1438-1446		
PATP (600 MPa-105°C)										
0 min	67	179	48	1.4	-0.1±0.2 <sup>AB</sup>	212	621	8	26	0.1±0.2 <sup>AB</sup>
0.5 min	348	1399	645	0.5	-0.7±0.2 <sup>BCD</sup>	938	6192	59	16	-0.1±0.2 <sup>AB</sup>
1 min	1325	2433	2851	0.5	-1.5±0.2 <sup>E</sup>	2216	10829	168	13	-0.9±0.0 <sup>CDE</sup>
2 min	1304	3226	2301	0.6	-2.5±0.3 <sup>F</sup>	4195	11146	620	7	-1.7±0.0 <sup>E</sup>
5 min	2369	4152	3016	0.8	-5.1±0.3 <sup>I</sup>	4919	31230	570	9	-3.4±0.2 <sup>G</sup>
8 min	1417	2779	2943	0.5	-5.8±0.3 <sup>J</sup>	4363	42730	657	7	-5.2±0.1 <sup>IJ</sup>
TP (105°C-0.1 MPa)										
0 min	51	260	103	0.5	0.2±0.2 <sup>A</sup>	8	20	22	0.3	0.3±0.2 <sup>A</sup>
2 min	140	229	184	0.8	0.2±0.0 <sup>A</sup>	63	271	241	0.3	0.3±0.2 <sup>A</sup>
5 min	1956	1524	969	2.0	0.2±0.2 <sup>A</sup>	190	680	309	0.6	0.3±0.1 <sup>A</sup>
30 min	608	1335	1411	0.4	-1.1±0.0 <sup>DE</sup>	583	2164	1554	0.4	-0.1±0.1 <sup>ABC</sup>
70 min	750	2501	2359	0.3	-2.5±0.1 <sup>F</sup>	743	2446	2516	0.3	-1.1±0.4 <sup>DE</sup>
120 min	1001	2884	1949	0.5	-3.9±0.1 <sup>GH</sup>	2641	9643	6334	0.4	-2.6±0.5 <sup>F</sup>
180 min	798	6725	4179	0.2	< D.L.	6430	28580	30803	0.2	-4.7±0.4 <sup>HI</sup>
240 min	1040	4888	3136	0.3	< D.L.	4571	46611	21886	0.2	-5.1±0.1 <sup>IJ</sup>
HPP (600 MPa-35°C)										
0 min	n/a	n/a	n/a	-	-0.1±0.1 <sup>AB</sup>	n/a	n/a	n/a	-	0.02±0.0 <sup>AB</sup>
5 min	n/a	n/a	n/a	-	0.2±0.0 <sup>A</sup>	n/a	n/a	n/a	-	0.1±0.1 <sup>AB</sup>
30 min	n/a	n/a	n/a	-	0.2±0.1 <sup>A</sup>	n/a	n/a	n/a	-	0.2±0.1 <sup>A</sup>
70 min	n/a	n/a	n/a	-	0.03±0.1 <sup>AB</sup>	n/a	n/a	n/a	-	0.3±0.1 <sup>A</sup>

< D.L. represents spore survivors under method detection limit (< 10<sup>2</sup> CFU/ml).

n/a: data not available. SIMCA could not differentiate the treated samples into distinctive groups.

Means with the same letter are not significantly different.

## FIGURE LEGENDS

**Figure 1.** Sample pressure (-----) and temperature ( ——— ) histories during single- (A) and double-pulse (B) treatment. Processing times include pre-process time in a conditioning bath and pressure chamber (A-B<sub>1</sub>), pressure come-up time (B<sub>1</sub>-C<sub>1</sub> and B<sub>2</sub>-C<sub>2</sub>), pressure holding time (C<sub>1</sub>-D<sub>1</sub> and C<sub>2</sub>-D<sub>2</sub>), and depressurization time (< 2 s). Glycol bath temperature was maintained at 105.5°C. Time pausing between two pulses (D<sub>1</sub>-B<sub>2</sub>) was kept at 1 min.

**Figure 2.** Soft independent modeling by class analogy on resistant property of untreated *Bacillus amyloliquefaciens* TMW 2.479 spores as influenced by different sporulation media (A) and different spore crop preparations using TSAYE as a sporulation medium (B)

**Figure 3.** Soft independent modeling by class analogy of different class projections of *Bacillus amyloliquefaciens* TMW 2.479 spores (NAYE grown) after pressure-assisted thermal processing at 600MPa-105°C (A) and thermal processing at 105°C-0.1 MPa (B).

**Figure 4.** Discrimination power plot in classification of pressure-assisted thermal processing treated *Bacillus amyloliquefaciens* TMW 2.479 spores grown on two different media.



**Figure 5.** Comparison of single- (SP) and double-pulse (DP) treatments on *Bacillus amyloliquefaciens* TMW 2.479 spores processed at equivalent holding time for 3 min; TSAYE grown spores (A) and NAYE grown spores (B).

**Figure 6.** Discrimination power plot in classification of *Bacillus amyloliquefaciens* TMW 2.479 spores grown on two different media after thermal processing at 105°C-0.1 MPa.

**Figure 7.** Comparison of discriminating power plot of *Bacillus amyloliquefaciens* TMW 2.479 spores grown on NAYE at 3 log reduction after TP (105°C, 0.1 MPa, 120 min) and PATP (600 MPa, 105°C, 5 min) treatments.

**Figure 8.** Cross-validated (leave-one-out) partial least squares regression plots for spore inactivation by pressure-assisted thermal processing; TSAYE grown spores (A) and NAYE grown spores (B).

**Figure 9.** Cross-validated (leave-one-out) partial least squares regression plots for spore inactivation by thermal processing; TSAYE grown spores (A) and NAYE grown spores (B).

**Figure 10.** Predicting microbial efficacy of double-pulse PATP treatment for *B. amyloliquefaciens* TMW 2.479 spore survivors based on single-pulse FT-IR spectra model. Predicted spore survivors by FT-IR spectra ( — ) and measured spore

survivors by standard plate count ( ◆ ); TSAYE grown spores (A) and NAYE grown spores (B). Refer to Figure 1B for nomenclature during the double-pulse treatment.

FIGURE 1

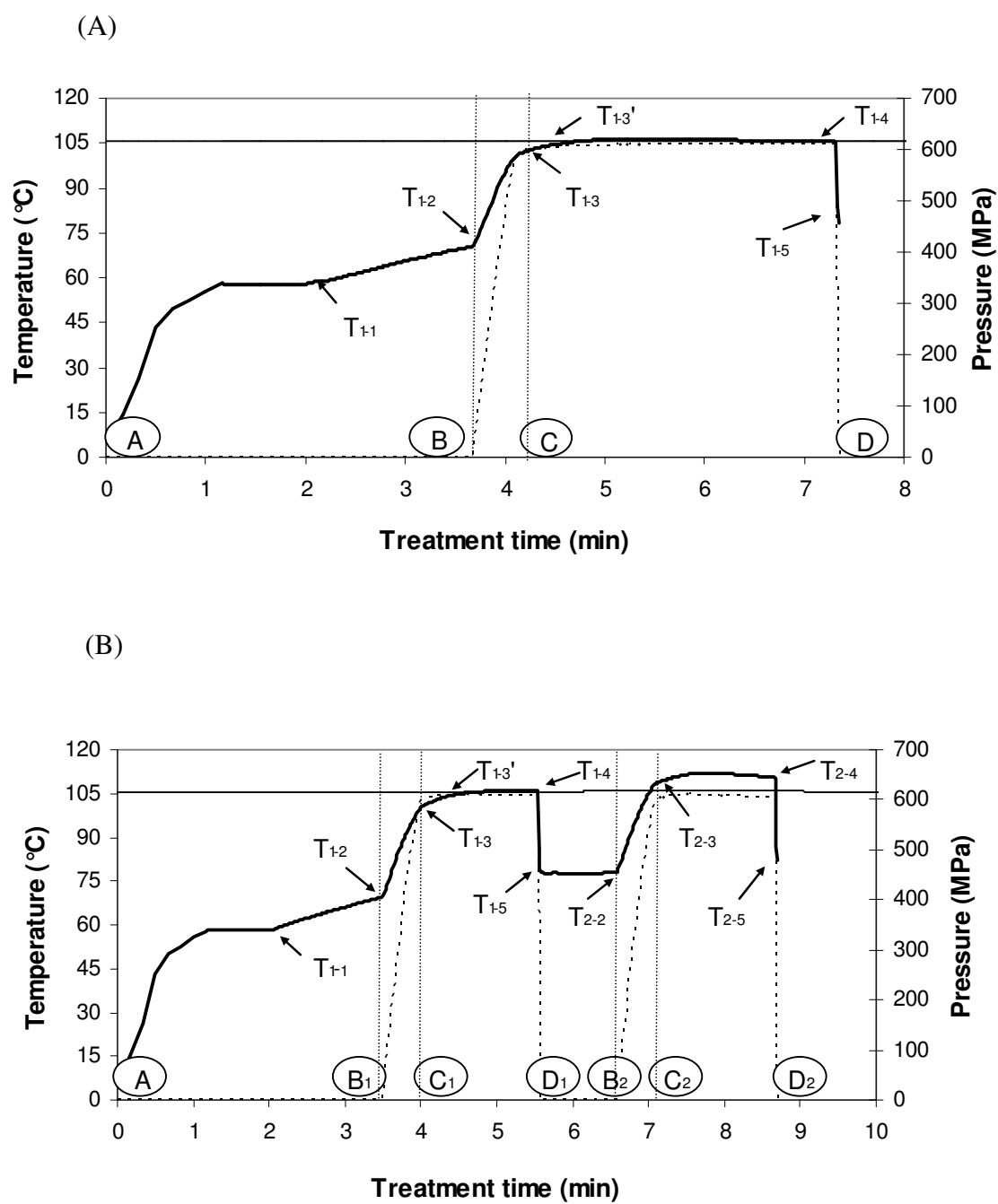
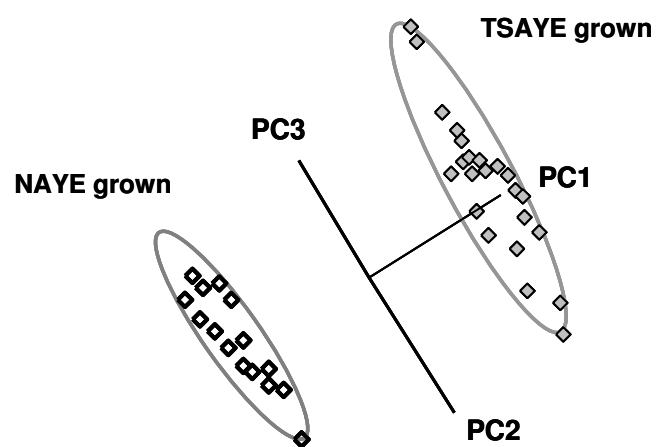


FIGURE 2

(A)



(B)

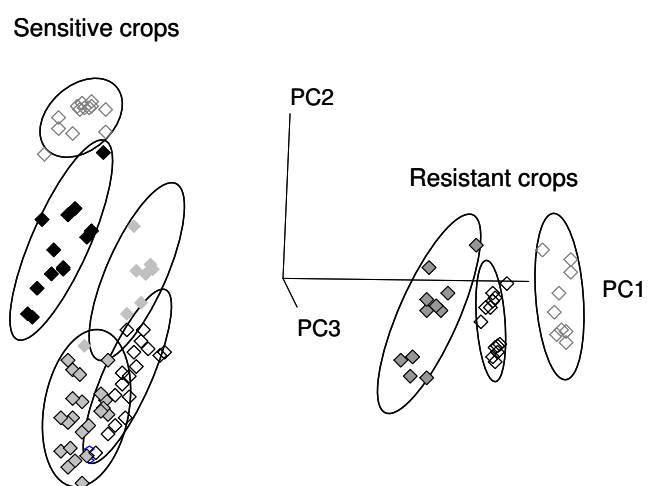
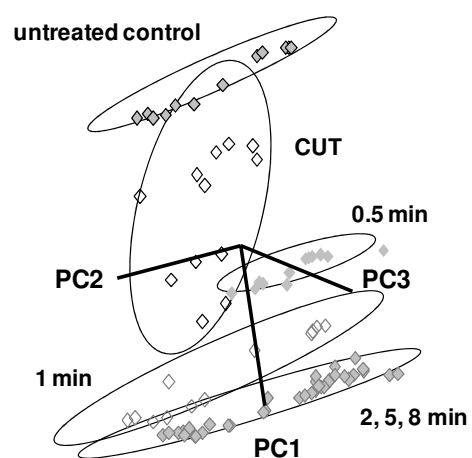


FIGURE 3

(A)



(B)

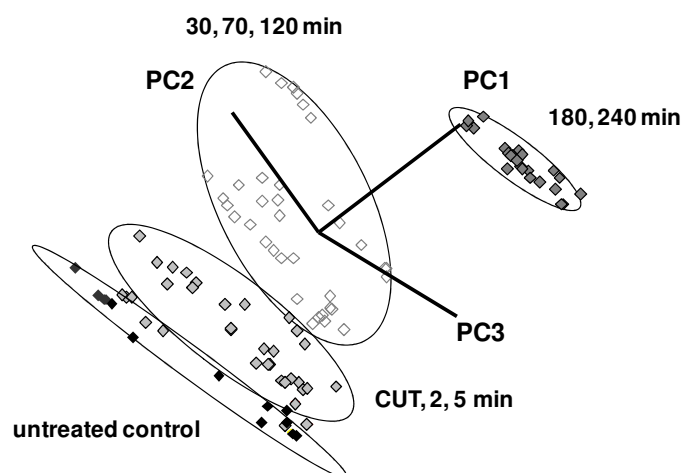


FIGURE 4

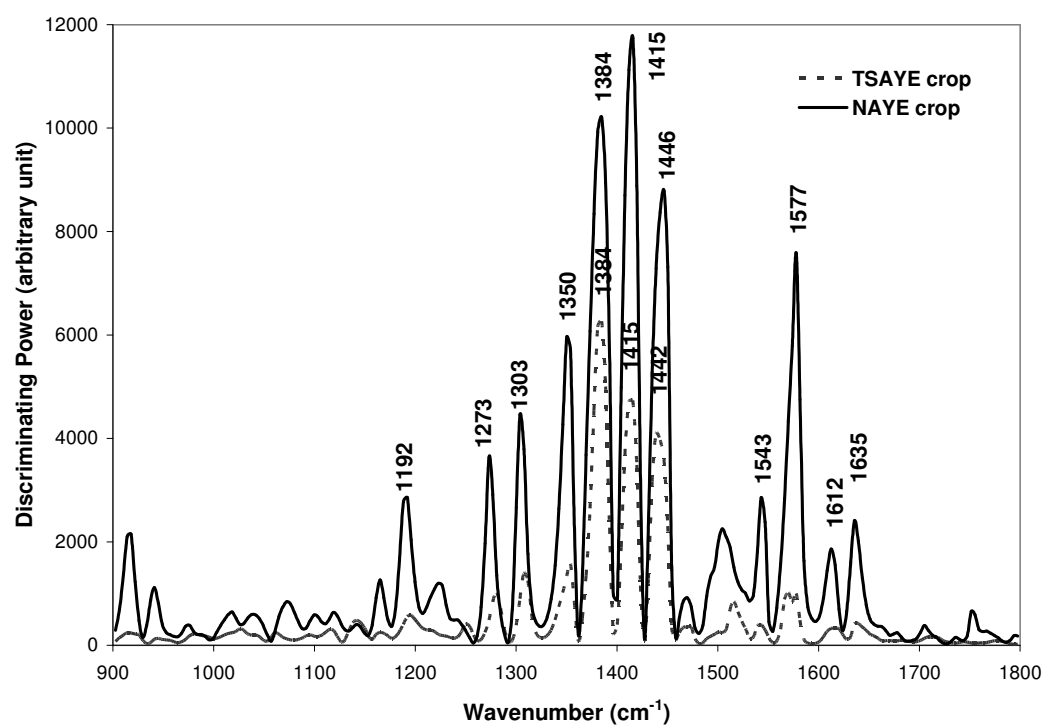


FIGURE 5

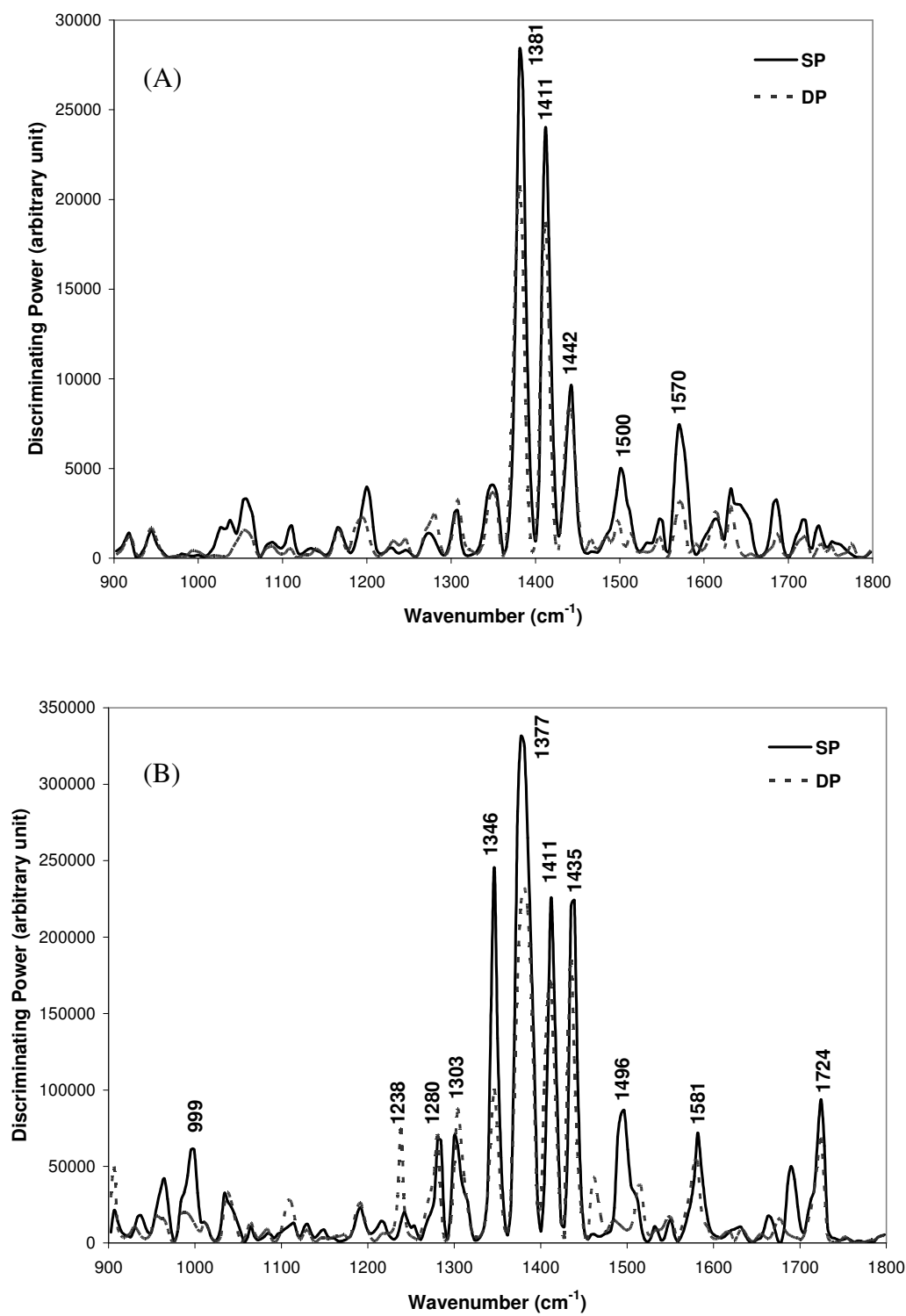


FIGURE 6

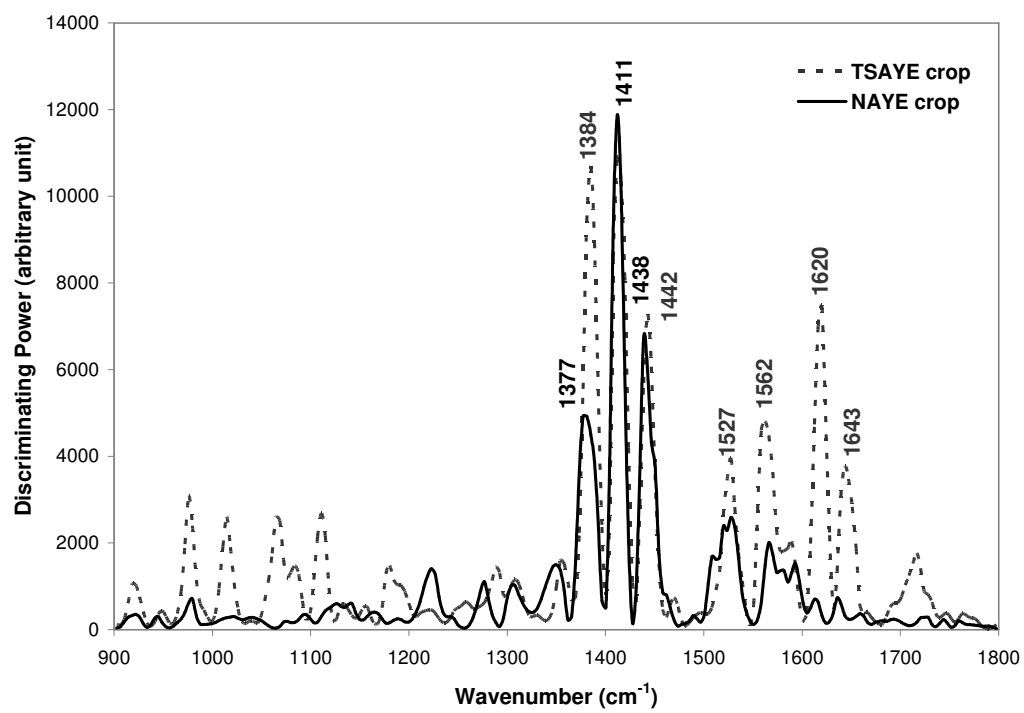




FIGURE 7

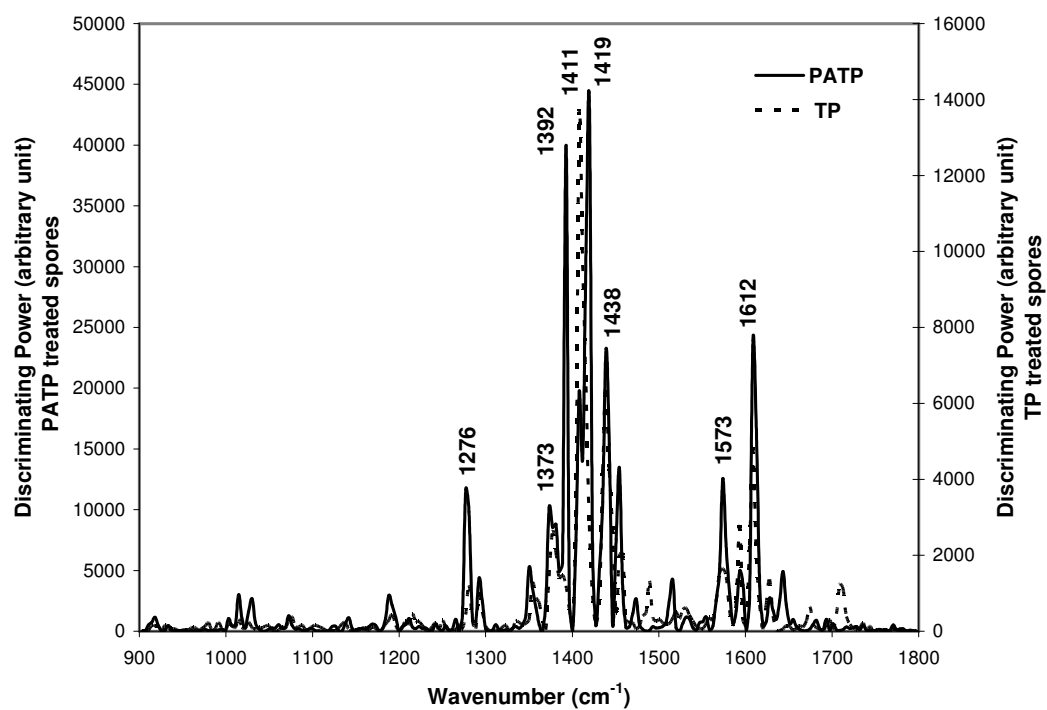
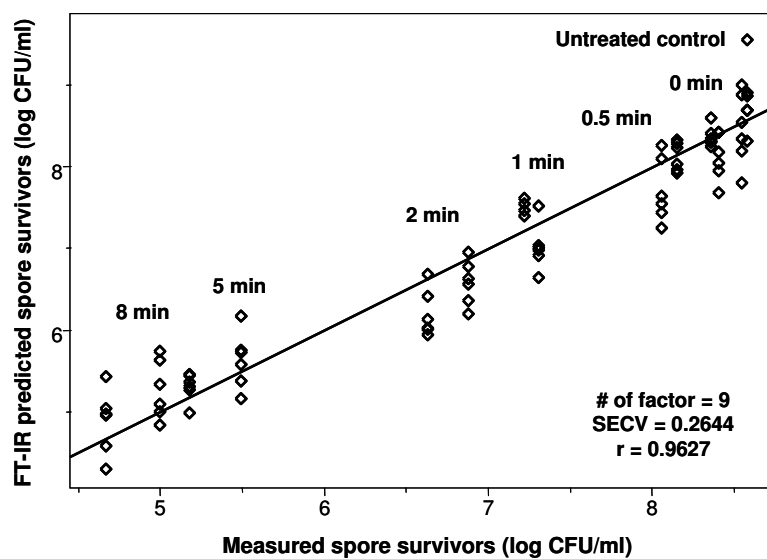


FIGURE 8

(A)



(B)

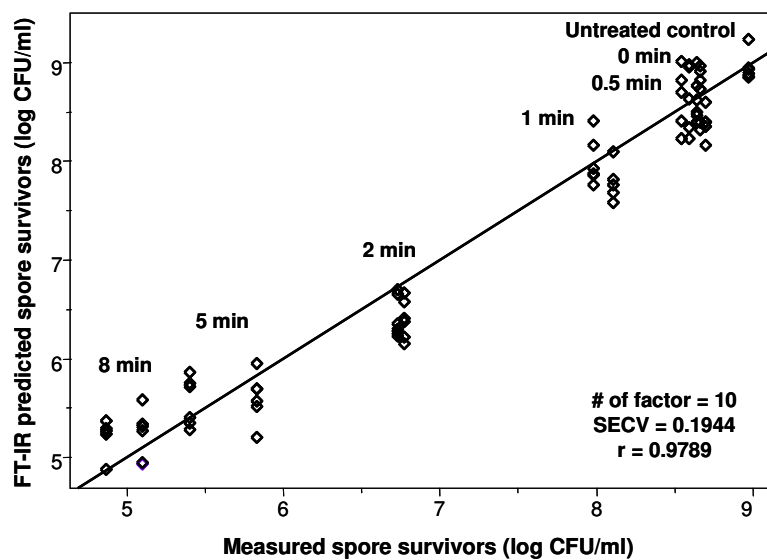


FIGURE 9

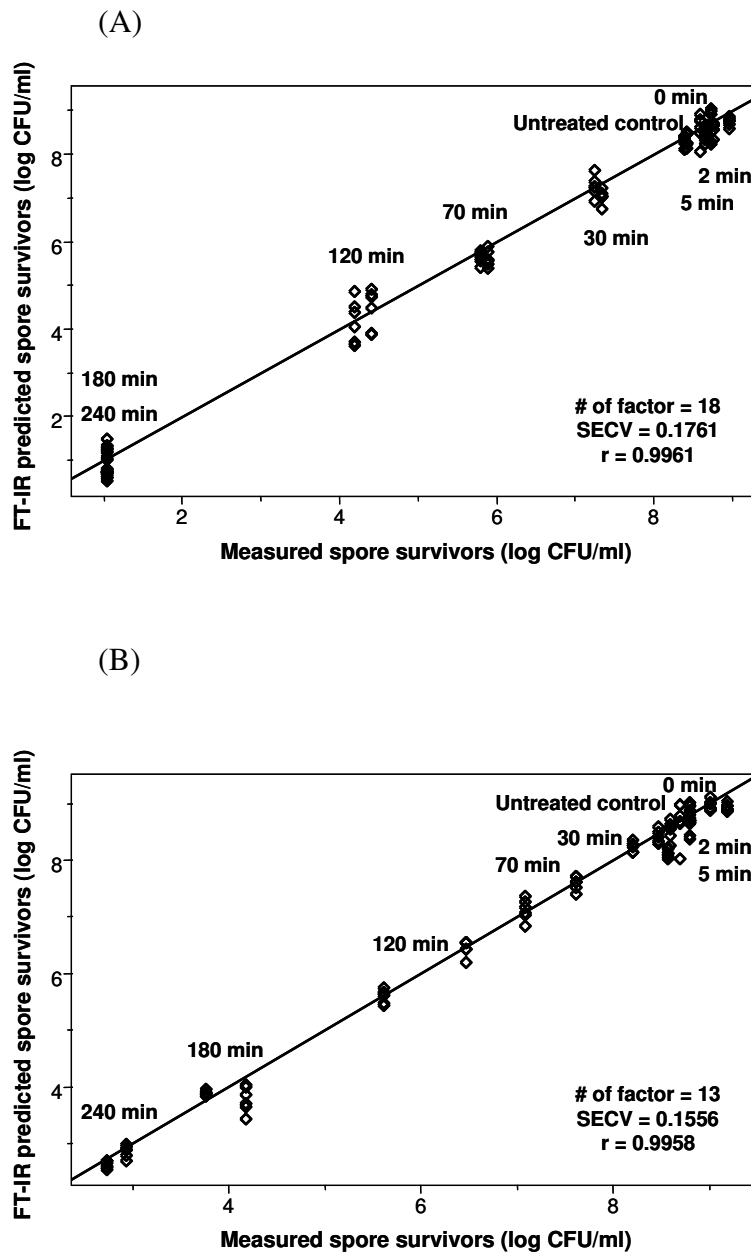
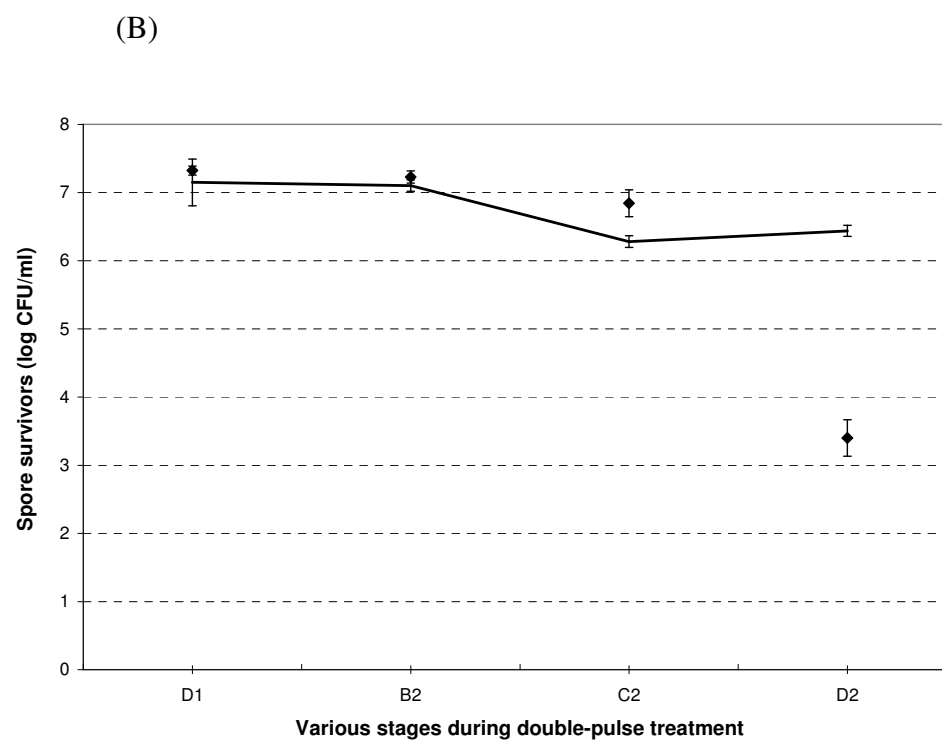
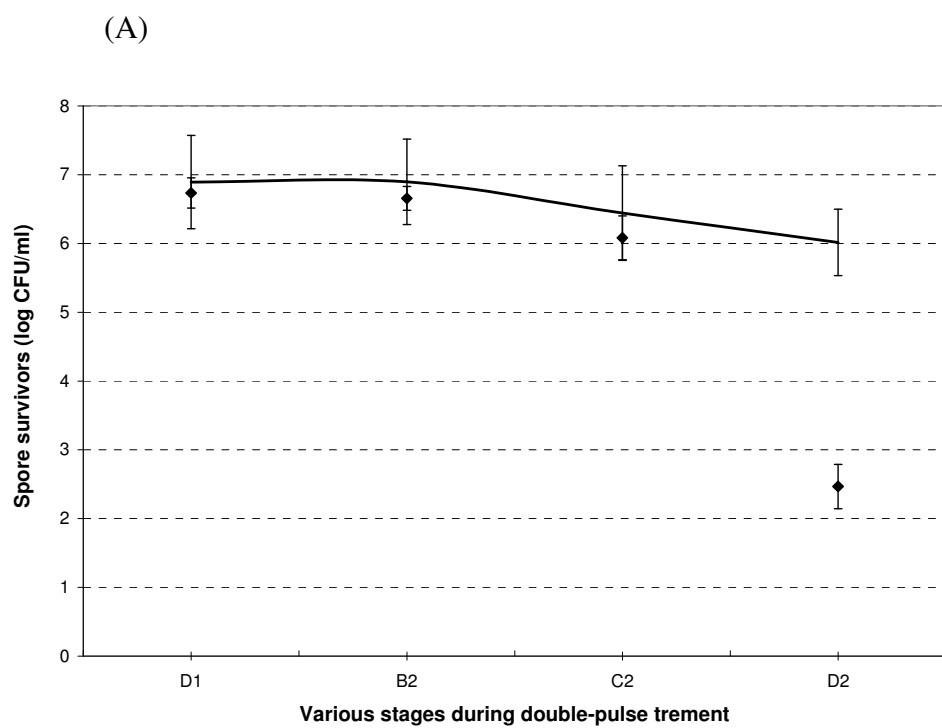


FIGURE 10



## CAPPS Project Progress Report

**Project Title:** Study on food constituent protective effects and spore injury during pressure-assisted thermal processing of a low-acid food

**Project Manager/Collaborator:** Dr. V.M. (Bala) Balasubramaniam and Dr. Ahmed E. Yousef, The Ohio State University

**Post-doctoral associate:** Dr. Hossein Daryaei and, The Ohio State University

**Description:** Pressure-assisted thermal processing (PATP) provides a viable alternative to conventional thermal sterilization of foods to produce commercially sterile shelf-stable low-acid products with superior quality. During PATP, foods are subjected to a combination of high pressure (500-700 MPa) and temperatures (90-121°C) for specific holding times. It is hypothesized that process lethality can be enhanced by addition of certain antimicrobial compounds to foods prior to PATP treatment.

This study will investigate combined pressure-thermal resistance of spores in the presence of selected antimicrobial compounds.

**Previous Work:** Our earlier CAPPS-funded work on sucrose esters (SE) showed some lethality enhancement when combined with PATP. Presence of 1% sucrose laurate enhanced the lethality of PATP against *Bacillus amyloliquefaciens* spores suspended in sterile deionized water (DIW) and mashed carrot by 0.55 to 1.2 log units, depending on the holding time applied. Population of viable spores in SE solutions decreased to <100 CFU/ml after 2 min treatment at 700 MPa and 105°C. On the other hand, addition of nisin (a heat and pressure stable bacteriocin) at concentrations of up to 2000 IU ml<sup>-1</sup> did not enhance the lethality of PATP under the experimental conditions tested.

**Related Work Elsewhere:** A number of researchers have reported the inactivation and growth inhibition effects of several antimicrobial compounds against various vegetative microorganisms and spores under moderate temperature and pressure conditions. Most of the compounds studied, showed an inhibitory effect (prevention of outgrowth and recovery during storage) and their effectiveness in enhancing PATP lethality against bacterial spores is yet to be investigated.

### **Progress to date:**

- **Combined effect of PATP and antimicrobial compounds on the inactivation of *B. amyloliquefaciens* Fad 82 spores in HEPES buffer (a screening study)**

The purpose of this research was to screen the lethal effect of PATP against *B. amyloliquefaciens* spores in the presence of the selected antimicrobial compounds suspended in 50 mM HEPES buffer. About 20 antimicrobial compounds were screened to date. The HEPES buffer was chosen due to its strong buffering capacity and minimal pH shift during PATP treatment. Unless specified, the compounds were examined at pH 7.0.

The stock solutions of antimicrobial compounds were freshly prepared at room temperature on the day of experiment and filter-sterilized using a membrane filter (0.22 µm) before adding to spore suspension.

- **Monoglycerides:** Monolaurin (C<sub>12:0</sub>), monomyristin (C<sub>14:0</sub>), monolinolein (C<sub>18:2</sub>) and monolinolenin (C<sub>18:3</sub>) were tested at concentration of 5 g/l. The stock solutions of monoglycerides were prepared in 95% propylene glycol.
- **Sodium salt preservatives:** Sodium benzoate and sodium nitrite were tested at concentrations of 0.5 and 2 g/l, respectively. The stock solutions were prepared in HEPES buffer.
- **Chelating agents:** Ethylenediaminetetraacetic acid (EDTA) was tested in the form of sodium salt (Na-EDTA) at concentration of 2 g/l. The stock solution of EDTA was prepared in HEPES buffer and its pH was adjusted to 7.0 by 10 M sodium hydroxide. In a separate trial, the lethal effect of 2 g/l EDTA at pH 5.0 combined with PATP treatment against spores was also tested.
- **Surfactants:** Sodium dodecyl sulfate (SDS) and saponin were tested at concentration of 2 g/l. the stock solutions were prepared in buffer.
- **Natural polymers:** Chitosan (a semi-synthetic polymer formed by alkaline deacetylation of chitin) was tested at concentration of 2 g/l and pH 6.0 and 5.0. It was insoluble at higher pH values. To prepare the stock solution, chitosan was initially dissolved in acetic acid at pH 5.0.
- **Antibiotics:** Tylosin solution was added to spore suspension at the level of 5 ml/l.
- **Bacteriocins:** Commercial pediocin (from *Pediococcus acidilactici*) and nisin (from *Lactococcus lactis*) were tested at concentrations of 5 µg/ml and 1 mg/ml (1000 IU/ml), respectively. The stock solutions were prepared in HEPES buffer at pH 7.0. Nisin was initially dissolved in 50% ethanol before adding to HEPES buffer.
- **Protein based:** Lactoferrin (an iron-binding milk protein) and lysozyme (an antibacterial enzyme) were tested at concentrations of 0.5 and 2 g/l, respectively.
- **Sucrose esters:** Sucrose stearate and sucrose palmitate were tested at concentration of 5 g/l.
- **Phenolic compounds of essential oils:** Eugenol, cinnamaldehyde, limonene and carvacrol at concentration of 0.5 g/l, and thymol at concentration of 0.1 g/l were tested. To prepare the stock solutions, Eugenol, cinnamaldehyde and limonene were dissolved in 50% ethanol, and carvacrol and thymol in 95% ethanol.

**Spore preparation and PATP treatment:** *Bacillus amyloliquefaciens* TMW 2.479 Fad 82 spores were used in this study, due to their high pressure-thermal resistance. The spores were prepared according to the method of Rajan *et al.* (2006) using nutrient agar as the sporulation medium supplemented with 0.6% yeast extract and 10 ppm MnSO<sub>4</sub>·H<sub>2</sub>O and stored in DIW (containing about 10<sup>9</sup> CFU/ml) at 4°C until used. For PATP treatment, appropriate aliquots of spore suspension were aseptically added to 50 mM sterile HEPES buffer containing antimicrobial compounds at selected concentrations (or without antimicrobial compounds as controls) in sterile pouches to obtain ~ 4.7 x 10<sup>8</sup> CFU/ml. The heat-sealed pouches were stored at 4°C for 1 h and treated in duplicate at 600 MPa and 105°C for 2 min in a high pressure microbial kinetic tester (PT-1, Avure Technologies Inc., Kent, WA) as described by Rajan *et al.* (2006). The 2-min treatment time at 600 MPa

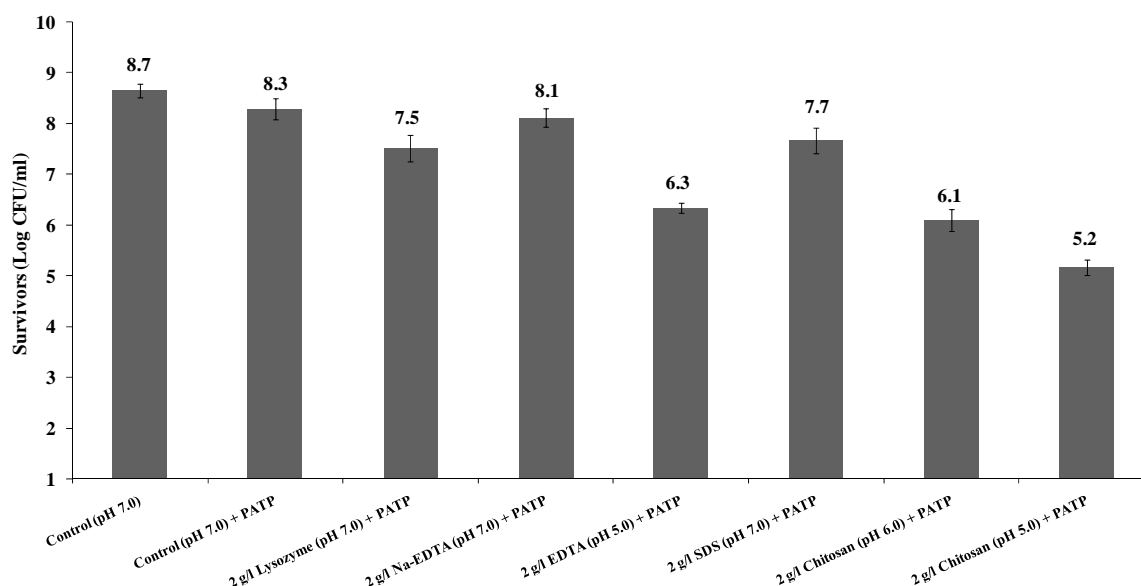
and 105°C was selected so that efficacy of antimicrobial compounds in enhancing PATP lethality against spores can be estimated. All experiments were independently repeated in a separate trial. PATP-treated and untreated samples were enumerated within 3 h after treatments.

### **Key results and observations:**

- Efficacy of 20 antimicrobial compounds in combination with PATP treatment have been tested.
- No noticeable changes in the pH of HEPES buffer was detected after PATP treatment.
- No considerable germination was detected during PATP treatment in any of samples with or without added antimicrobial compounds. This was confirmed by applying a heat shock at 80°C for 15 min to a proportion of the first dilution during viable count procedure and comparing the result with non-heat-shocked spore count.
- PATP treatment of *B. amyloliquefaciens* spores at 600 MPa and 105°C in 50 mM HEPES buffer (pH 7.0) in the absence of antimicrobial compounds did not result in any significant inactivation (only 0.4 log reduction was achieved).
- Among the different chemical and natural antimicrobial compounds tested in this screening study, the following compounds showed promising potential (**Figure 1**)
  - lysozyme
  - SDS at PH 7.0,
  - EDTA at pH 5.0 and
  - chitosan at pH 5.0 and 6.0 were capable to significantly enhance the PATP lethality.

The initial population of *B. amyloliquefaciens* spores in buffer was reduced by approximately 1.2 and 1.0 log units in the presence of 2 g/l lysozyme and SDS, respectively at pH 7.0; and 2.6 log units in the presence of 2 g/l chitosan at pH 6.0. Addition of 2 g/l chitosan to spore suspension at pH 5.0 resulted in a 3.5 log reduction under PATP conditions. Under the experimental conditions, 2 g/l sodium salt of EDTA (Na-EDTA) at pH 7.0 did not sufficiently enhance the PATP lethality; however, the presence of EDTA at pH 5.0 reduced the spore count by approximately 2.4 log units.

- It is interesting to note that certain antimicrobial compounds, including monoglycerides (especially monolaurin) previously reported effective during thermal processing were not sufficiently enhanced PATP lethality against *B. amyloliquefaciens* spores, possibly due to differences in their mechanisms of action against bacterial spores during PATP.



**Fig.1** Enhancing PATP (600 MPa and 105°C, 2 min) lethality against *B. amyloliquefaciens* spores in 50 mM HEPES buffer in the presence of selected antimicrobial compounds. The initial inoculum level was  $\sim 4.7 \times 10^8$  CFU/ml. Bar graphs represent the mean of spore counts obtained from analyses of duplicate pouches of two PATP trials.

## Conclusions:

*B. amyloliquefaciens* is a highly pressure-thermal resistance spore. The findings to date demonstrate that the efficacy of PATP against these spores could be enhanced by addition of certain antimicrobial compounds to spore suspension prior to treatment. This could possibly provide a method to process foods at less severe heat and pressure conditions and thus preserve quality attributes.

## Ongoing research:

This research will continue to:

- Investigate the efficacy of selected pre-treatments to sensitize the spores prior to PATP treatment. This will include evaluating the effect of pre-treatment temperature on pressure-heat resistance of spores in the presence of antimicrobial compounds.
- Identify similar promising compounds (enzymes, surfactants and natural polymers) and other potential natural antibacterial compounds such as fermented pollen, grapefruit extract and liquid smoke and CO<sub>2</sub> that may enhance the lethality of PATP.
- Examine combinations of certain antimicrobial compounds for enhancing PATP lethality. Individual or combinations of antimicrobial compounds showing greater synergy with PATP will be selected to reduce PATP severity.



**Bibliography:**

Rajan, S., Ahn, J., Balasubramaniam, V.M., and Yousef, A.E. 2006. Combined pressure-thermal inactivation kinetics of *Bacillus amyloliquefaciens* spores in mashed egg patties. *Journal of Food Protection* 69(4), 853-860.

**Relevant Publications from past CAPPS funded efforts:**

Ratphitagsanti, W., Ahn, J., Balasubramaniam, V.M., and Yousef, A.E. 2009. Influence of pressurization rate and pressure pulsing on the inactivation of *Bacillus amyloliquefaciens* spores during pressure-assisted thermal processing. *Journal of food protection* 72(4), 775-782.

Ratphitagsanti, W., De Lamo-Castellvi, S., Balasubramaniam, V.M., and Yousef, A.E. 2009. Efficacy of pressure-assisted thermal processing, in combination with organic acids, against *Bacillus amyloliquefaciens* spores suspended in deionized water and carrot puree. *Journal of food protection* (Submitted).